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Abnormal Expression of CD44 Variants in the Exfoliated Cells in the Feces of Patients With Colorectal Cancer

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See editorial on page 1333.

Background & Aims: Recent investigations have shown that CD44 variant exons are frequently overexpressed in human colorectal adenocarcinoma. The aim of this study was to investigate abnormal expression of the CD44 gene in exfoliated cells from patients with colorectal cancer. *Methods:* Exfoliated cells in feces from 25 patients with colorectal cancer before and after surgery and from 15 healthy volunteers were analyzed. CD44 standard, variant 6, and variant 10 messenger RNA (mRNA) expressions were examined in the exfoliated cells in feces by using reverse-transcription polymerase chain reaction followed by Southern hybridization with exon-specific probes. *Results:* CD44 standard mRNA was detected in all samples before and after surgery and in all healthy volunteers. CD44 variant 6 and variant 10 mRNA were detected in 17 of 25 patients (68%) and 15 of 25 patients (60%), respectively, in individual feces obtained before surgery. CD44 variant 6 mRNA and variant 10 mRNA were detected in postoperative samples in 3 of 25 patients (12%) and 7 of 25 patients (28%), respectively. Fifteen of 17 patients who were positive for CD44v6 based on preoperative fecal samples became negative after surgery (88.2%). Similarly, 12 of 15 patients who were CD44v10 positive in preoperative fecal samples were negative postoperatively (80%). *Conclusions:* These results suggest that analysis of CD44 variant expression in the exfoliated cells in feces can provide a noninvasive diagnostic test for colorectal cancer.

A widely distributed transmembrane glycoprotein, CD44, plays an important role in lymphocyte homing,[1] lymphocyte activation,[2] cell-cell adhesion,[3] and cell-matrix adhesion.[4,5] CD44 is known to be linked with the development and spread of carcinoma including metastasis.[6] CD44 molecules show various isoforms that are generated from a single gene by alternative messenger RNA (mRNA) splicing, in which 10 different variant exons (v1–v10) are involved, and by posttranslational modifications.[7,8] Several such CD44 variants (CD44v) have been detected in various human tumor cell lines and human tumor tissues.[9–18] Abnormal retention of intron 9 in CD44 gene transcripts also has been shown in several tumors.[19,20] Although the functions of CD44 variant isoforms have not been clarified, CD44 variants are considered to play an important role in tumor growth and metastasis.

The standard isoform of CD44 (CD44s) is present in almost all normal cells, whereas other variant isoforms are expressed in a tissue-specific manner. Immunohistochemical studies have revealed that CD44s is expressed only in the proliferative zone of the crypts, whereas expression of CD44 variants was very weak or undetectable in normal human colonic mucosa.[21] Wielenga et al. reported that expression of v7–v10 was weakly detected, whereas expression of v3–v6 was not detectable in normal colorectal mucosa.[22] In contrast to this restricted expression of CD44 in the normal colorectal mucosa, colorectal carcinoma frequently shows overexpression of CD44s and several CD44 variants.

Immunohistochemical studies on bladder cancer with several monoclonal antibodies to many variant isoforms have shown differences in the location of the corresponding CD44 epitopes between normal and neoplastic bladder mucosa. The CD44v6 protein is confined to the basal layers of normal epithelium but is not detectable in superficial epithelial cells. In bladder cancer tissues, strong membranous immunostaining for variant CD44 molecules was seen both in the basal cells of the malignant epithelium and in its more superficial cells.[23] Immunocytochemistry[23] and reverse-transcription polymerase chain reaction (RT-PCR)[19] for exfoliated urinary cells showed that cancer cells in the urine from patients with bladder cancer possessed CD44v6 protein and the mRNA, but exfoliated normal epithelial cells and lymphocytes did not.

Abbreviations used in this paper: CD44s, CD44 standard; CD44v, CD44 variant; RT-PCR, reverse-transcription polymerase chain reaction; TBS, Tris-buffered saline.

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Studies of the expression of CD44 mRNA in normal colorectal mucosa and neoplasms, which suggested an important role of CD44 in the progression of colorectal neoplasms, have also been reported.[24,25]

Thus, we examined whether abnormal expression of the CD44 gene in colorectal cancer could be detected in the exfoliated cells in naturally voided feces using the RT-PCR method as a novel approach for molecular diagnosis.

Materials and Methods

Patients

A total of 25 patients who had histologically confirmed colorectal cancer and 15 normal healthy volunteers were examined. Fourteen male and 1 female healthy volunteer had no remarkable abnormality on colonoscopic examination. The median age of the volunteers was 32 years (range, 27–42). The characteristics of the patients are summarized in Table 1. All patients underwent surgical resections of their primary tumors between December 1995 and September 1997. The median age of the patients was 65 years (range, 45–76). Sixteen patients were male and 9 patients were female. The locations of the tumors were rectum in 12 patients, sigmoid colon in 7, transverse colon in 2, ascending colon in 2, and cecum in 2. The clinical stages of the patients according to Dukes' classification were 8 at Dukes' A, 4 at B, and 13 at C.

Isolation of Cellular Debris in the Feces

Fecal samples were obtained from 25 patients with colorectal cancer both before and 1–2 months after surgical resection of their primary tumors. The preparation of each sample was conducted with some modification of the method described by Albaugh et al.[26] Approximately 5 g of the naturally voided feces was used for isolation of the exfoliated cells. Feces were collected in Stomacher Lab Blender bags (Stomacher, Seward, England) and were stored for 6–7 hours at 4°C until preparation. Feces were homogenized with a buffer (200 mL) consisting of 1 g/L $NaHCO_3$, 500 U/L penicillin G, 500 mg/L streptomycin, 1.25 mg/L amphotericin B, and 50 mg/L gentamicin by using the Stomacher 400T Lab Blender (Stomacher) at the lowest speed for 1 minute. Each homogenized fecal sample was centrifuged at 800*g* for 15 minutes, and then the supernatant was removed. The precipitant was suspended in physiological saline, and the suspended samples were placed on 60% Percoll (Pharmacia, Uppsala, Sweden) at a

Table 1. Clinicopathologic Characteristics of Patients

Patient no.	Age (*yr*)/sex	Tumor			Tumor invasion				Dukes' stage	RT-PCR detection of CD44v6 and v10 in exfoliated cells in feces			
										CD44v6		CD44v10	
		Location	Size (*mm*)	Histology	Depth	LY	V	N		Preoperative	Postoperative	Preoperative	Postoperative
1	69/F	Sigmoid	75 × 65	Well	SS	−	−	−	B	+	−	+	−
2	68/M	Ascending	60 × 63	Moderately	SE	+	+	+	C	−	−	−	−
3	53/M	Sigmoid	37 × 30	Moderately	PM	+	−	+	C	+	−	+	−
4	63/M	Sigmoid	70 × 60	Well	SS	+	−	+	C	+	−	+	−
5	69/M	Rectum	30 × 18	Moderately	A	+	−	−	B	−	−	−	−
6	61/M	Transverse	18 × 13	Well	SM	−	−	−	A	−	−	−	−
7	76/M	Rectum	75 × 45	Well	SE	−	−	+	C	−	−	−	−
8	66/M	Rectum	18 × 15	Well	PM	+	+	−	A	+	−	+	−
9	49/M	Rectum	35 × 32	Well	PM	−	−	+	C	+	−	−	−
10	53/F	Rectum	15 × 15	Well	M	−	−	−	A	−	−	−	−
11	50/M	Transverse	40 × 35	Moderately	SS	+	+	+	C	−	+	−	+
12	63/F	Sigmoid	21 × 20	Well	SS	+	+	+	C	+	+	+	+
13	68/F	Cecum	22 × 16	Well	SM	−	−	−	A	+	−	+	−
14	73/F	Rectum	40 × 35	Moderately	SS	+	−	+	C	+	−	−	−
15	71/F	Rectum	35 × 25	Well	SM	−	−	−	A	−	−	−	+
16	51/F	Sigmoid	25 × 22	Well	PM	+	−	−	A	+	−	+	+
17	45/M	Rectum	36 × 28	Well	A	+	+	+	C	+	−	+	−
18	70/M	Sigmoid	53 × 39	Moderately	SS	+	−	+	C	+	−	+	−
19	71/M	Rectum	45 × 40	Moderately	PM	−	+	−	A	+	+	+	+
20	56/F	Cecum	80 × 40	Moderately	SS	+	+	−	B	+	−	+	−
21	67/M	Sigmoid	36 × 26	Moderately	SS	+	+	+	C	+	−	+	−
22	64/M	Rectum	35 × 30	Well	PM	−	−	−	A	+	−	+	−
23	65/F	Rectum	45 × 45	Well	SS	+	+	+	C	+	−	+	−
24	60/M	Ascending	35 × 25	Moderately	SS	−	−	−	C	−	−	−	−
25	70/M	Rectum	60 × 60	Moderately	SS	−	−	−	B	+	−	+	−

NOTE. Immunohistochemistry was performed for patients 1–5.
Well, well-differentiated tubular adenocarcinoma; Moderately, moderately differentiated tubular adenocarcinoma; LY, lymphatic vessel invasion; V, vascular invasion; N, lymph node metastasis; M, mucosal; SM, submucosal; PM, muscularis propria; SS, subserosal; A, adventitia.

proportion of 4:6 by volume and centrifuged at 400*g* for 15 minutes at room temperature. After centrifuge, the lower part of the aqueous phase, which contained the cellular component, was collected and laid on 70% Percoll at a proportion of 3:7 by volume and centrifuged at 300*g* for 15 minutes at room temperature. The entire aqueous phase was collected and suspended in the saline and then centrifuged. The supernatant was removed, and the precipitant was stored at −80°C until mRNA extraction.

Extraction of mRNA and PCR

The mRNA was extracted from the cellular debris in the feces prepared by the aforementioned procedure using a Micro Fast-Track (Invitrogen, Carlsbad, CA), and complementary DNA (cDNA) was synthesized using a cDNA Cycle kit (Invitrogen). The synthesized cDNA was amplified by PCR. The PCR conditions were as follows: 94°C for 5 minutes and 85°C for 1 minute, during which time *Taq* polymerase was added (hot start), followed by 35 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. The primers used were SP1, 5′-TGGATCACCGACAGCACAGACAGA-3′ and SP2, 5′-GATGCCAAGATGATCAGCCATTCTGGAAT-3′, and all the inserted variant forms and the standard portion of CD44 exons could be amplified (Figure 1).

Southern Blot Hybridization and Chemiluminescent Detection

Ten microliters of the PCR products was electrophoresed in a 1.2% agarose gel and transferred to a Hybond N$^+$ nylon membrane (Amersham, Buckinghamshire, England) with 0.4N NaOH solution overnight and hybridized with the exon-specific probes for CD44s, exon 11 (v6), and exon 15 (v10). Each probe was made by amplifying the TA plasmid clones containing the CD44 variant exons from the ZR75-1 cell line or standard exon from human peripheral blood leukocytes using the related primers listed below. These were labeled with peroxidase using the enhanced chemiluminescence Direct Nucleic Acid Labeling Kit, and the signals on the membrane were detected chemiluminescently using an enhanced chemiluminescence detection system (Amersham). The conditions for hybridization, washing, and detection were those recommended by the manufacturer's protocol. The primers used were SE×16,5′-AGACCAAGACACATTCCA-3′; AE×17,5′TGTCCTTATAGGACCAGA-3′; Sv2,5′-TTGATGAGCACTAGTGCTACAGCA-3′; Av2,5′-CATTTGTGTTGTTGTGTGAAGATG-3′; Sv6,5′-GGCAACTCCTAGTAGTACAACG-3′; Av6,5′-CAGCTGTCCCTGTTGTCGAATG-3′; Sv10,5′-GATGTCACAGGTGGAAGAAGAGAC-3′; and Av10,5′TTCCTTCGTGTGTGGGTAATGAGA-3′ (Figure 1).

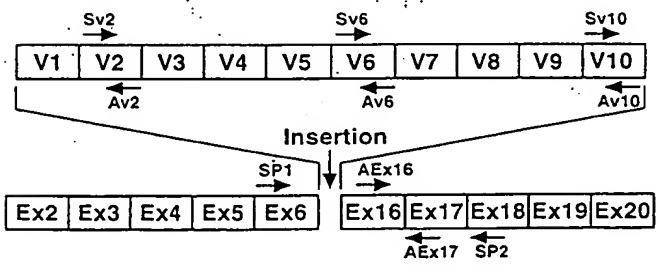

Figure 1. Map of CD44 gene products, showing exons and primers used in this study. RT-PCR for each sample was conducted using Sp1 and Sp2 primers. The probes for CD44 standard, v6, and v10 were made by PCR using primers of SE×16 and AE×17, Sv6 and Av6, and Sv10 and Av10, respectively.

Immunohistochemistry

In 5 patients, presence of total CD44 protein and CD44v6 protein was analyzed by immunohistochemistry (Figures 2 and 3). Cryostat tissue sections (6 μm) were fixed in ice-cold methanol for 20 minutes, washed in Tris-buffered saline (TBS), and preincubated with 20% normal rabbit serum in TBS. After washing with TBS, separate sections were incubated with the primary antibodies F10-44-2 (3.3 μg/mL) and 2F10 (10 μg/mL), which recognize the epitopes of the CD44s portion and CD44v6 portion, respectively, in 1% normal rabbit serum diluted with TBS at 4°C overnight followed by 30 minutes at 37°C the next morning. As shown in Figure 1, CD44v exons are alternatively spliced into the mRNA strand corresponding to the standard form. Therefore, the positive staining with F10-44-2 indicates the protein expression of total CD44 isoforms including CD44v and CD44s. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol, and the sections were incubated with a 1:400 dilution of biotinylated anti-mouse immunogloblin (Dako, Japan, Kyoto, Japan) at 37°C for 60 minutes, followed by incubation with horseradish peroxidase–conjugated ABComplex (Dako) at room temperature for 60 minutes. The peroxidase activity was developed with 3,3-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) for 20 minutes, and the reaction was stopped in water. All sections were counterstained with Mayer's hemalum.

Results

First we studied a case of sigmoid colon cancer (Figure 2). RT-PCR followed by Southern blotting analysis revealed that the amplified CD44 gene products from the tumor tissue (Figure 2*B*, lane 4) showed a long intense smear with a v6 probe, but those from the normal tissue of the same patient gave only two discrete bands (Figure 2*B*, lane 3). In the exfoliated cells in the fecal sample from this patient, several bands were seen in the preoperative fecal sample (Figure 2*B*, lane 1) but no band was obtained in the postoperative one (Figure 2*B*, lane 2). Whereas the standard form of the CD44 gene was clearly expressed in all the specimens examined (Figure 2*A*), in the tumor tissue, more intensive and larger molecules were found compared with the PCR products from normal tissue (Figure 2*A*, lane 3 vs. lane 4). For the fecal sample, the preoperative one (Figure 2*A*, lane 1) had a thicker standard band and several additional variant bands, but the postoperative one (Figure 2*A*, lane 2) had only one discrete standard band.

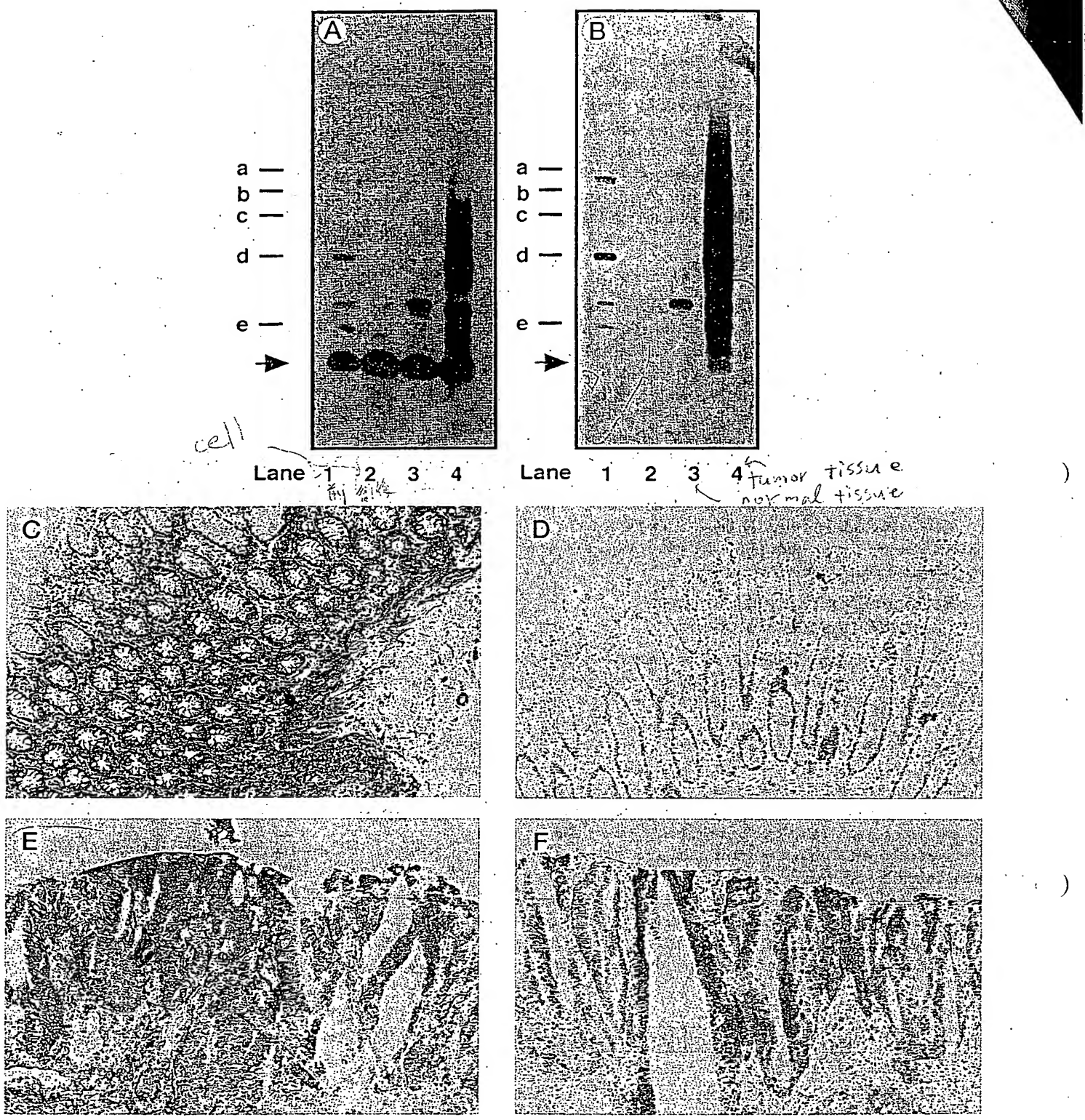

Figure 2. RT-PCR–Southern blot analysis of fecal samples and immunohistochemical staining with monoclonal antibodies to total CD44 and CD44v6 protein in a surgically resected specimen in the same patient (patient 1 in Table 1). (*A*) Southern blot analysis of RT-PCR products from fecal samples using a CD44s exon–specific probe. *Lane 1,* fecal sample before surgical resection of the primary tumor; *lane 2,* fecal sample after surgical resection of the primary tumor; *lane 3,* resected normal colonic mucosa; *lane 4,* resected cancerous lesion. (*B*) Southern blot analysis of RT-PCR products from the fecal samples using a CD44v6-specific probe in the same patient. *Lane 1,* fecal sample before surgical resection of the primary tumor; *lane 2,* fecal sample after surgical resection of the primary tumor; *lane 3,* resected normal colonic mucosa; *lane 4,* resected cancerous lesion. Molecular weight markers a, b, c, d, and e denote 1.35-, 1.08-, 0.87-, 0.60-, and 0.31-kilobase pairs, respectively. Immunohistochemical detection of CD44 expression: (*C*) normal colonic mucosa stained for total CD44, (*D*) normal colonic mucosa stained for CD44v6, (*E*) colonic adenocarcinoma stained for total CD44, and (*F*) colonic adenocarcinoma stained for CD44v6.

Immunohistochemical studies with monoclonal antibodies to an epitope of the standard form of CD44 (F10-44-2) (Boehringer Mannheim, Mannheim, Germany) and to that of CD44v6 (2F10) (R & D Systems, Abingdon, England) showed differences in the locations of the corresponding CD44 epitopes between normal and neoplastic colonic mucosa. F10-44-2 showed that in normal colonic epithelium, the total CD44 protein can be detected in the crypts and is also present in stromal cells and smooth muscle cells (Figure 2*C*). Interestingly, a weaker staining was observed at the more superficial surface of mucosa. A very weak expression of CD44v6 was confined to the basal layers of normal epithelium (at the base of the crypts) but was not detectable in surface epithelium or stromal cells (Figure 2*D*). In neoplastic colonic specimens, strong membranous immunostaining for total CD44 and for CD44v6 was seen in almost all tumor tissues (Figure 2*E* and *F*).

We performed an immunohistochemical study for total CD44 and CD44v6 in 4 additional patients (Figure 3*A–P*). The staining pattern of the immunohistochemistry was the same as that of the first patient mentioned above. We also performed the RT-PCR with SP1 and SP2 primers in the exfoliated cells in feces from these patients before and after surgical resection of the primary tumors. Hybridization with v6- and v10-specific probes showed that v6 and v10 mRNA expression was detected in two samples (patients 3 and 4; lanes 3 and 5 in Figure 3*R* and *S*), respectively, from the patients with sigmoid colon cancer. The other two preoperative samples (patients 2 and 5) from the patients with ascending colon cancer and rectal cancer did not have v6 and v10 expression (lanes 1 and 7 in Figure 3*R* and *S*). No CD44v6 and v10 mRNA were detected in any of the 4 patients after surgery (lanes 2, 4, 6, and 8 in Figure 3*R* and *S*).

In total, we examined exfoliated cells in fecal samples pre- and postoperatively from 25 patients with colorectal cancer. In all exfoliated cells in both the preoperative and postoperative fecal samples, CD44 mRNA was detectable by RT-PCR and Southern blot analysis. In the preoperative exfoliated cells in the feces, hybridization with CD44s exon–specific probe showed the clear bands of the standard isoform in all samples and numerous additional larger-molecular-weight bands, which showed smear or multiple bands, in all samples except for two (11 and 24), in which only one standard isoform band was detected (Figure 4*A*). In the postoperative exfoliated cells in the fecal samples, the patterns of the CD44 isoforms were different from the preoperative ones. Although the bands of the standard isoform were clearly detected similarly to the preoperative samples, the numbers and sizes of additional larger-molecular-weight bands in the postoperative samples were smaller than those in the preoperative samples in most patients (Figures 2*A*, 3*Q*, and 4*A* and *B*).

Hybridization with a v6-specific probe showed that v6 mRNA expression was detected in 17 of 25 fecal samples (68%) obtained preoperatively (Figures 2*B*, 3*R*, and 5*A*), whereas CD44v6 mRNA was detected in only 3 patients after operation (Figures 2*B*, 3*R*, and 5*B*). Among 17 patients whose preoperative fecal samples were positive for CD44v6, 15 patients (88.2%) became negative for CD44v6 in the postoperative samples (Figures 2*B*, 3*R*, and 5*A* and *B*). A similar pattern was observed with the detection of CD44v10 expression. Expression of CD44v10 mRNA was detected in 15 of 25 preoperative samples (60%) (Figures 3*S* and 6*A*). In 12 of those 15 patients (80%), v10 expression was not detected in the samples obtained after surgical resection of tumors (Figures 3*S* and 6*A* and *B*). In 1 patient (11), CD44v6 and CD44v10 mRNA were detected only in the postoperative samples (Figures 5 and 6).

There was no relationship between clinical stage (Dukes' stage) or degree of differentiation of the tumors and the detection of CD44v6 and v10 mRNA in the exfoliated cells of the preoperative feces. The frequency of positivity of CD44v6 or v10 CD44 mRNA in the exfoliated cells in the preoperative feces was comparable between the early stage (Dukes' A: CD44v6, 62.5%; CD44v10, 62.5%) and advanced stage (Dukes' B/C: CD44v6, 70.6%; CD44v10, 58.8%) (Table 1). CD44v6 and CD44v10 mRNA in the exfoliated cells in the preoperative feces were more detectable in patients with tumors of the rectum or sigmoid colon than in patients with tumors of other sites. CD44v6 mRNA was detected in 8 of 12 patients with rectal cancer (66.6%) and in all 7 patients with sigmoid colon cancer in the exfoliated cells in the preoperative feces (Table 1). In the exfoliated cells in the postoperative feces, CD44v6 and CD44v10 mRNA was detected in 1 of 12 patients with rectal cancer (8.3%) and in 1 of 7 patients with sigmoid colon cancer (14.2%), respectively. A similar observation was seen in the detection of CD44v10 mRNA (Table 1).

We examined CD44s, CD44v6, and CD44v10 mRNA expression in the exfoliated cells in feces obtained from 15 normal healthy volunteers known not to have any malignancies including colorectal cancer. The CD44 standard isoform was detected in the exfoliated cells in feces of all normal healthy volunteers (Figure 7*A*), whereas CD44v6 and CD44v10 mRNA were detected in the exfoliated cells in the feces of only 1 normal healthy volunteer (lane 3 in Figure 7*B* and *C*).

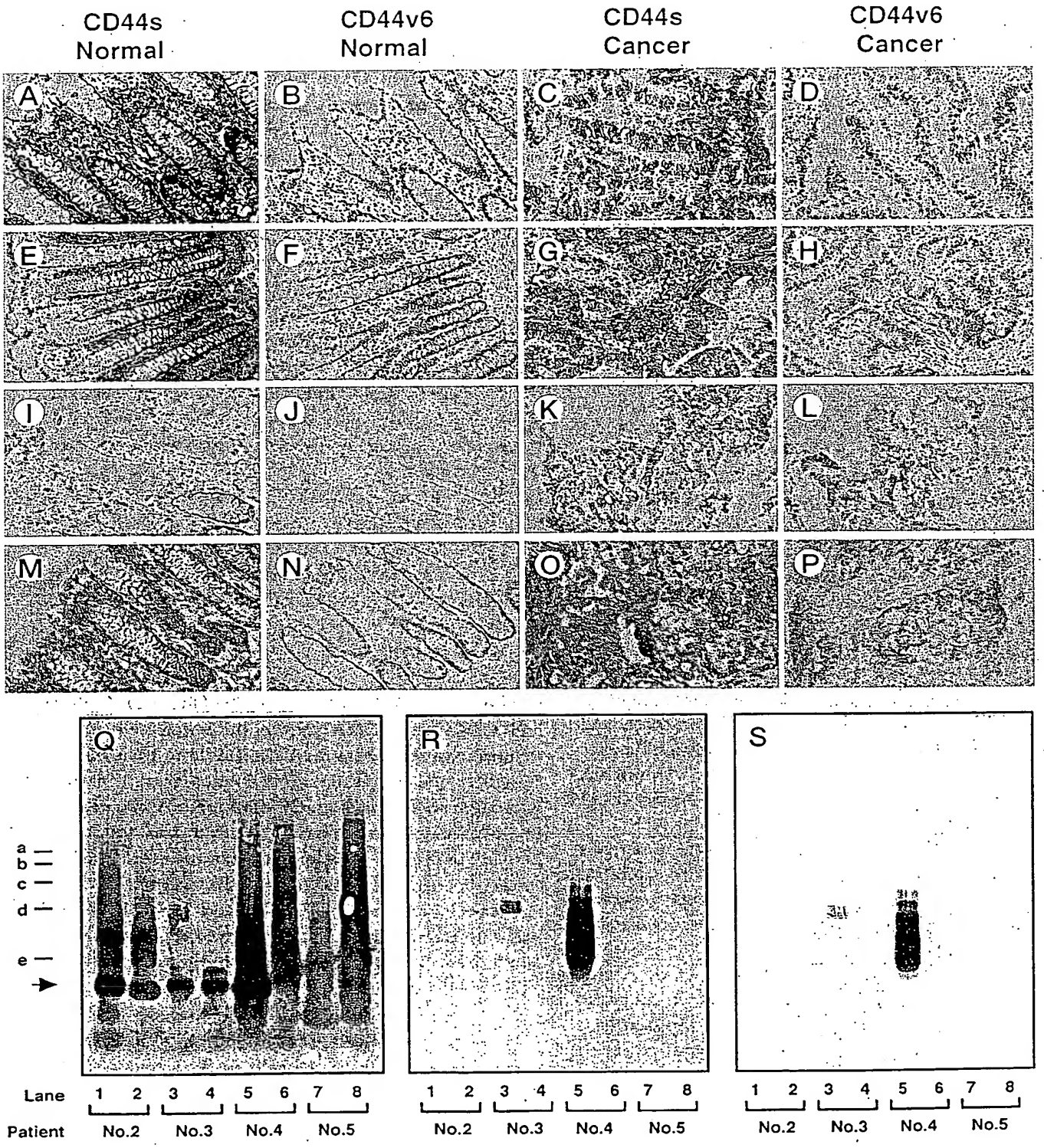

Figure 3. (*A–P*) Immunohistochemical staining with monoclonal antibodies to total CD44 and CD44v6 protein in surgically resected specimens from 4 patients with colorectal cancer (patients 2 [*A–D*], 3 [*E–H*], 4 [*I–L*], and 5 [*M–P*] in Table 1). (*Q*) RT-PCR–Southern blot analysis of exfoliated cells in feces before surgery (*lanes 1, 3, 5,* and *7*) and after surgery (*lanes 2, 4, 6,* and *8*) in each patient using a CD44s exon–specific probe (patient 2, *lanes 1* and *2;* patient 3, *lanes 3* and *4;* patient 4, *lanes 5* and *6;* and patient 5, *lanes 7* and *8*). (*R*) RT-PCR and Southern blot analysis of the same filter using a CD44v6-specific probe. (*S*) RT-PCR and Southern blot analysis of the same filter using a CD44v10-specific probe.

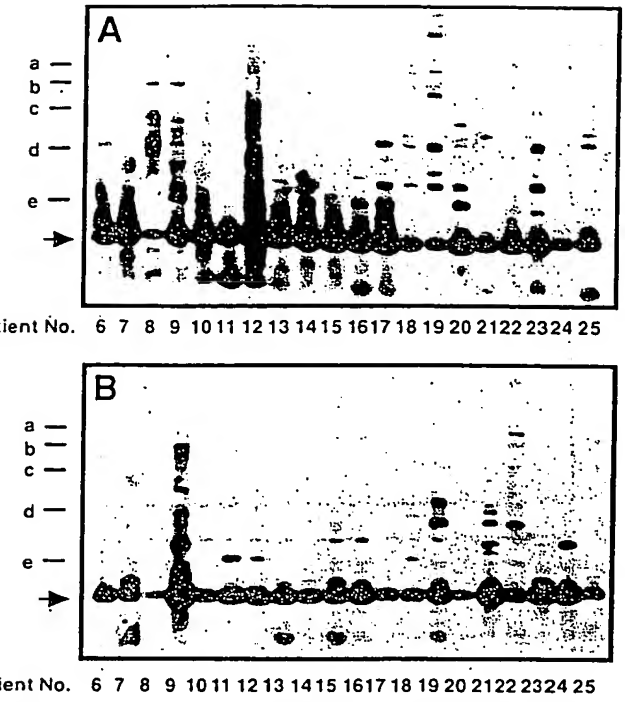

Figure 4. Southern blot analysis of RT-PCR products from individual samples of exfoliated cells in fecal samples from patients with colorectal cancer (patients 6–25 in Table 1). PCR products obtained with CD44 standard primers (Sp1 and Sp2, see Figure 1) were resolved on an agarose gel, and after Southern blotting, the filter was hybridized with a CD44-exon standard probe (see Materials and Methods). (*A*) Exfoliated cells in fecal samples from patients before surgical resection of the primary tumors. (*B*) Samples from the corresponding patients after surgical resection of the primary tumors. Molecular weight markers a, b, c, d, and e denote 1.35-, 1.08-, 0.87-, 0.60-, and 0.31-kilobase pairs, respectively.

Discussion

For mass screening of cancer, simple and noninvasive methods for detection of cancer are important. In clinical practice for the mass screening of colorectal cancer, testing the fecal occult blood is widely used. In this study, we clearly show that detection of a gene expression in the cellular debris of a small amount of naturally voided feces was possible by a combination of proper preparation of the fecal samples, RT-PCR, and Southern blot hybridization.

Although the sample size analyzed was small, some interesting observations were made. First, the pattern of distribution of the molecular weight of CD44 isoforms was different between the preoperative and postoperative samples. Compared with the postoperative samples, the preoperative samples show gross overexpression of several large-molecular-weight variants and a more complex pattern. Second, most patients whose preoperative samples were positive for expression of the CD44v exon (v6) became negative for it after surgical removal of their tumors. A similar pattern was observed in the detection of v10 expression. These differences between the preoperative and postoperative samples were likely a result of the change in components of the cellular debris of feces by removal of the primary tumor, i.e., the preoperative samples contained carcinoma cells but the postoperative samples did not. We believe that these results reflect the quantitative and qualitative differences in CD44 expression between normal colorectal mucosa and colorectal carcinoma. We noticed that the RT-PCR data are highly concordant with the immunologic results. The immunologic data revealed that in normal tissues, cells at the basal crypts were very weakly stained, but the superficial epithelial cells were completely negative for v6 antibody. F10-44-2 antibody also showed a strong reaction at the basal crypts, but at the more superficial surface, a weaker reaction was observed. On the other hand, in tumor tissue, strong staining was observed even at the superficial part of the tumor tissue both for F10-44-2 and 2F10 antibody. The immunohistochemistry using a monoclonal antibody that recognized CD44v6 revealed that the lymphocytes in the colonic mucosal layer were negative for CD44v6. Combining the immunohistological data with the information from the RT-PCR, we speculate

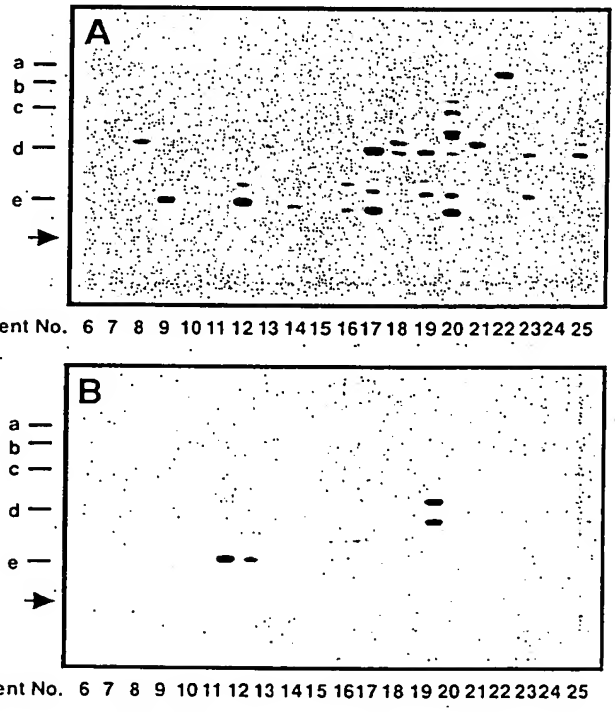

Figure 5. The same filter as in Figure 4 was rehybridized with a CD44v6-specific probe. (*A*) Exfoliated cells in fecal samples from patients before surgical resection of the primary tumors. (*B*) Samples from the same patients after surgical resection of the primary tumors. Molecular weight markers a, b, c, d, and e denote 1.35-, 1.08-, 0.87-, 0.60-, and 0.31-kilobase pairs, respectively.

that, in normal conditions, the exfoliated cells consist of the superficial epithelial cells and some lymphocytes, which are usually lacking in v6 antigen. However, we were able to detect v6 mRNA in the exfoliated cells from the patients with colorectal cancer, because the cancer cells that are in direct contact with the stool and are also positive for v6 antigen may be easily peeled off into the colonic lumen. These results suggest that cancer cells exfoliated in naturally voided feces still have deranged expression of the CD44 gene, strongly supporting the possibility of a clinical application to identify colorectal carcinoma by the detection of this expression. However, the source of CD44v6 transcripts is essentially unknown because we have not succeeded in identifying colonic cancer cells cytologically. The source of the mRNA of CD44 variants in the fecal samples may have originated from activated lymphocytes or tumor infiltrated lymphocytes.

Matsumura et al. reported significant CD44 mRNA expression in the exfoliated cancer cells in the urine for the diagnosis of bladder carcinoma.[17,19] However, it remains uncertain whether expression of CD44 is a suitable biomarker for molecular diagnosis of colorectal cancer in terms of its sensitivity and specificity. Wielenga et al. reported that CD44v6 protein expression was

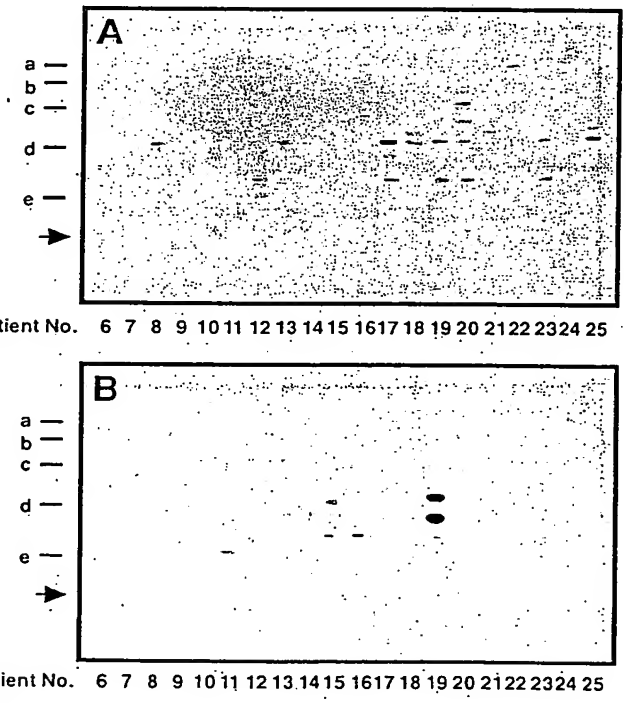

Figure 6. The same filter as in Figure 4 was rehybridized with a CD44v10-specific probe. (*A*) Exfoliated cells in fecal samples from patients before surgical resection of the primary tumors. (*B*) Samples from the same patients after surgical resection of the primary tumors. Molecular weight markers a, b, c, d, and e denote 1.35-, 1.08-, 0.87-, 0.60-, and 0.31-kilobase pairs, respectively.

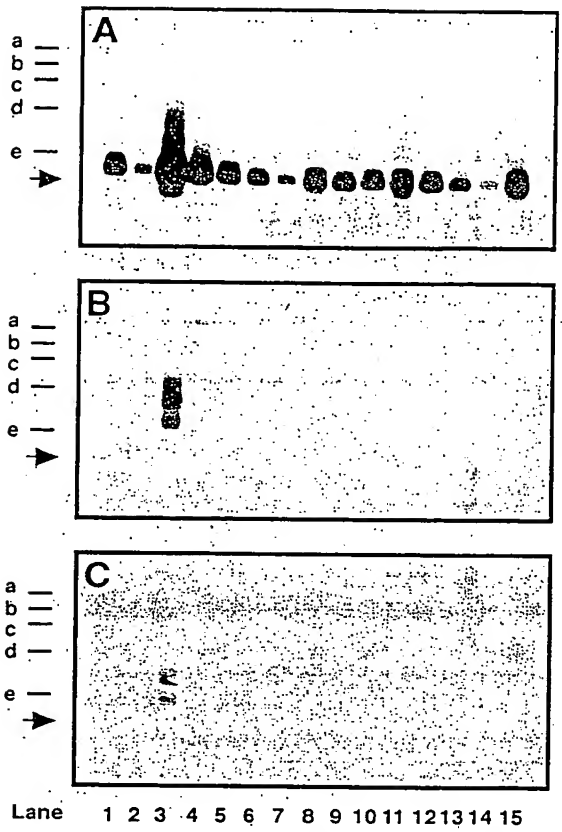

Figure 7. Southern blot analysis of RT-PCR products from individual samples of exfoliated cells in fecal samples from 15 normal health volunteers. (*A*) Hybridization with a CD44s exon–specific probe. (*B*) Hybridization with a CD44v6-specific probe. (*C*) Hybridization with a CD44v10-specific probe. The filter used in each hybridization was identical.

detectable by immunohistochemistry in none of the normal colonic mucosa but in 9%, 45%, and 68% of early and late adenomas and invasive carcinoma, respectively.[22] Thus, we have to keep in mind the existence of false positivity and false negativity and the limitations in the diagnostic property for genetic diagnosis using detection of CD44 variant expression. CD44v6 mRNA was not detectable in the exfoliated cells in preoperative feces in 8 of 25 (32%) patients with colorectal cancer in the present study. The reasons for the negativity were: (1) no expression of the CD44 variants in the tumor tissue, (2) no exfoliated tumor cells in the fecal sample, and (3) lack of sensitivity of the present methodology despite the presence of variant-positive tumor cells in the fecal sample. On the contrary, CD44v6 mRNA was detected in the exfoliated cells in the postoperative feces in 3 of 25 patients (12%) with colorectal cancer. Additionally, CD44v6 mRNA was detected in 1 of 15 normal healthy volunteers. The false positivity is likely caused by the coexistence of colonic adenomas, inflammation of colonic mucosa accompanied with activated lymphocytes, and

regeneration of colonic mucosa. Three false positives in the CD44v6 mRNA expression in exfoliated cells in postoperative feces were probably a result of regeneration or inflammation of colonic mucosa after surgery.

We analyzed only one fecal sample, approximately 5 g in weight, in the preoperative and postoperative period from each patient. Thus, it was uncertain whether there was intrapatient variation concerning CD44v6 and CD44v10 mRNA expression under the same conditions. However, we speculate that the frequency of detection of CD44v6 and v10 mRNA may increase with an increased number of fecal samples from a given patient, and this may be helpful in increasing sensitivity. To confirm the diagnostic value of detecting CD44 expression in the exfoliated cells in feces for colorectal cancer, further studies with a larger sample size including a normal control are required.

The positive rates of CD44v6 in the exfoliated cells of preoperative feces in the patients with rectal and sigmoid colon cancer and other sites of the colon were 78.9% (15 of 19 patients) and 33.3% (2 of 6 patients), respectively. We believe that exfoliated cells from a tumor localized in the rectum and sigmoid colon are more viable than those from tumors localized in a more oral site of the colon. Additionally, it is interesting that CD44v6 or CD44v10 was detectable in the exfoliated cells in preoperative feces from the patients with an early stage of colorectal cancer. There were 8 patients whose tumors were at the early clinical stage (Dukes' stage A). CD44v6 mRNA was detected in the exfoliated cells in preoperative feces in 5 of 8 (62.5%) patients with such early-stage colorectal cancer, and 4 of 5 patients who were positive for CD44v6 mRNA preoperatively became negative for it postoperatively. A similar observation was seen with the detection of CD44v10 mRNA in the exfoliated cells in feces of the patients with such early-stage colorectal cancer. Based on these results, the methods described can be used to identify rectosigmoid cancer even at an early stage.

We did not analyze CD44 expression in exfoliated cells from the patients with nonneoplastic diseases such as Crohn's disease or ulcerative colitis. Rosenberg et al. reported that increased expression of CD44v6 was detected immunohistochemically in colonic epithelial crypt cells in 23 of 25 ulcerative colitis samples compared with 3 of 18 samples of Crohn's disease.[27] RT-PCR analysis of CD44 expression in exfoliated cells in feces should be performed in patients with inflammatory bowel disease.

In conclusion, we showed that exfoliated cells in the feces could be an important material for the molecular diagnosis of colorectal cancer. The methods described may open the way to future investigation of molecular diagnosis of colorectal cancer.

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